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Do we need another semi-automated approach to measure muscle fibre cross-sectional area?

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The recent publication by Gilda, et al (2021) (*1*) is yet another example of a semi-automated pipeline to analyse muscle fibre cross-sectional area (FCSA), a topic that has seen 13 methodology papers published in the last three years (*2-16*). While the experimental data used to validate their methodological approach is of high quality there are several points worth comment.

Firstly, there is a lack of discussion comparing their approach to other available options; regrettably, only four of the recently published data pipelines do so either (*2, 3, 5, 15*). The major benefit suggested by Gilda, et al. is that their approach is more user-friendly compared to other programs, and was more efficient compared with manual analysis (using the same software); both statements are subjective. Where one might argue that the use of confocal microscopy (as in this paper) provides an unnecessary level of detail for simply calculating FCSA, it requires a degree of training, which they highlight as inconvenient when assessing other semi-automated software packages. Additionally, Imaris is a commercial image analysis software, a point not made by the authors, a potentially unnecessary expense compared to recent methods that are freely available (*2-16*).

Moreover, the authors reason that it is necessary to measure all available fibres in a muscle biopsy to accurately reflect FCSA, while simultaneously arguing that areas of tissue may be rejected from analysis if necessary. This dichotomy is equally baffling and inaccurate. While a large sample size may be required to detect small changes or infrequent events, it is statistically inefficient to count all fibres within a muscle cross section; with an appropriate unbiased, random sampling regime it is possible to provide statistically robust estimates of both average muscle FCSA and fibre size distributions (*17*). Additionally, any whole tissue approach risks overlooking important structural heterogeneities, where phenotypically/ anatomically defined compartments within muscles may be more appropriate (*18, 19*).

Importantly, measuring FCSA alone is not novel (*2-16, 20*). The free software packages are not only able to semi-automatedly segment muscle fibre boundaries, but in some instances semi-automatically assign muscle fibre phenotype (13 of 16), incorporate colocalization of nuclei and capillaries (10 of 16), and in one case includes the option to model oxygen transport kinetics (*13*). Where the authors have differentially identified calpain-1 shRNA transfected fibres for FCSA analysis, this process is like identification of muscle fibre types based on immunoreactivity, and again this process is not discussed in the wider context of the field of semi-automated processing.

Skewness is not a new statistic in this field, as fibre size increases in a geometric manner; a statement about needing different statistical tests depending on its value requires justification and examples.

We bring these points to your attention in the hope that future semi-/fully-automated software packages are appropriately verified against competing options in order to substantiate claims of superiority. We suggest new methodological studies should focus on speed of processing, the biological imperative to identify and integrate histologic primitives (e.g. nuclei and

capillaries) with quantitative outputs, and development of sequential pipelines like those of FEA modelling approaches (*13*) in order to substantially advance the field.

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